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n-Paraffin Assimilating Yeasts Grown at 37°C

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Abstract

Among 284 *n*-paraffin assimilating yeast strains isolated from soil, 21 strains showed good growth in *n*-paraffin medium at comparatively high temperature (37°C) in shaking culture. One of them gave 95.5% cell yield against *n*-paraffin added to medium and the cells had a protein content of 61.8% in jar fermenter studies. Taxonomical studies showed that all the 21 strains belonged to the genus *Candida*, including one new species. These are 1 strain of *C. kofuensis* nov. sp., 12 strains of *C. tropicalis*, 2 strains of *C. albicans*, 2 strains of *C. solani*, 1 strain of *C. krusei*, 1 strain of *C. intermedia*, 1 strain of *C. rugosa* and 1 strain of *C. parapsilosis*.

Introduction

It is very advantageous to be able to cultivate microorganisms at high temperature for industrial microbial cell production, because a large difference in temperature between cooling water and fermentation medium can remove efficiently the heat formed during fermentation. It is especially profitable in the case of cell production from *n*-paraffin, because hydrocarbon fermentation liberates far more heat than conventional fermentation using carbohydrate as substrate.[1)]

Up to date, many *n*-paraffin assimilating yeast strains have been isolated from natural sources or found in stock culture collections, and their abilities to assimilate *n*-paraffins have been reported. However, cultivation temperatures were around 30°C, and there are few reports dealing with the importance of high cultivation temperature in hydrocarbon fermentation. Although the temperature of industrially available cooling water depends on the place and the season, we consider that there is sufficient difference in temperature between cooling water and fermentation medium to cool the medium during fermentation at 37°C.

Iizuka *et al.*[2,3)] reported that *Candida rugosa* JF-114 showed the best growth in hydrocarbon medium at 28°C among strains isolated by them. In our studies, this strain also gave the best growth of our stock cultures in *n*-paraffin medium at 30°C, but there was no growth at 37°C. Therefore we tried to isolate yeast strains from soil which can grow at 37°C in *n*-paraffin medium. Among 284 strains isolated by us, 21 strains showed almost the same or better growth in *n*-paraffin medium at 37°C in shaking culture than *C. rugosa* JF-114 at 30°C. In jar fermenter studies of the 21 strains, the cell yield against *n*-paraffin added to the medium, and protein content of the cells were determined. One of them gave good cell yield and high protein content.

Taxonomical studies showed that all the 21 strains belonged to the genus *Candida*, including one new species. In this paper, the results of isolation, cultivation and taxonomical studies are described.

Materials and Methods

Microorganisms *Candida rugosa* JF-114[2,3] was supplied as a good *n*-paraffin assimilating yeast by The Institute of Applied Microbiology and preserved in our laboratory. *n*-Paraffin assimilating yeasts grown at 37°C were isolated from soil samples in Japan by enrichment culture methods or the sprinkled soil plate method.

***n*-Paraffin and media** A mixture of *n*-paraffins produced by Nikko Petrochemicals Co., Ltd., was used. Table 1 gives the composition of the *n*-paraffin mixture. The media used for isolation and cell production culture are shown in Table 2.

Isolation and culture conditions The enrichment culture method by shaking culture, and the sprinkled soil plate method differed from those described by Takahashi, Kawabata and Yamada[4] only in the isolation media and cultivation temperature. Cultivation temperature was 37°C in our studies. Another enrichment culture method using a jar fermenter was also carried out. Fifty soil samples were simultaneously added to a 10 *l* jar fermenter and incubated for several days at 37°C. After two subcultures, *n*-paraffin assimilating yeasts were isolated by the usual monocolony isolation method.

Growth studies were carried out in shaking flasks or in 10 *l* jar fermenters. The reciprocal shakers were run at a rate of 120 rpm at 37°C. Jar fermenters were operated with an agitation speed of 800 rpm, an aeration rate of 1 vvm, and pH control with aqueous ammonia.

Table 1. Composition of *n*-paraffin mixture.

n-Nonane	0.1 %	*n*-Octadecane	12.8 %
n-Decane	0.2	*n*-Nonadecane	10.3
n-Undecane	0.4	*n*-Eicosane	7.1
n-Dodecane	0.9	*n*-Heneicosane	4.9
n-Tridecane	2.7	*n*-Docosane	2.9
n-Tetradecane	8.1	*n*-Tricosane	1.4
n-Pentadecane	15.0	*n*-Tetracosane	0.5
n-Hexadecane	16.7	*n*-Pentacosane	0.2
n-Heptadecane	15.8		

Table 2. Composition of isolation and production media.

Isolation medium		Production medium	
n-Paraffin	3.00 % (v/v)	*n*-Paraffin	3.00 % (v/v)
$(NH_4)_2SO_4$	0.20 % (w/v)	$(NH_2)_2CO$	0.38 % (w/v)
K_2HPO_4	0.08 %	$(NH_4)_2HPO_4$	0.20 %
KH_2PO_4	0.02 %	KCl	0.08 %
$MgSO_4 \cdot 7H_2O$	0.02 %	$MgSO_4 \cdot 7H_2O$	0.06 %
NaCl	0.02 %	NaCl	0.01 %
$CaCl_2 \cdot 2H_2O$	0.05 %	$FeSO_4 \cdot 7H_2O$	0.001 %
$FeSO_4 \cdot 7H_2O$	0.005 %	$ZnSO_4 \cdot 7H_2O$	0.001 %
$(NH_4)_2MoO_4$	0.005 %	Molasses	0.10 %
Chloramphenicol	0.002 %		

a) Composition of *n*-paraffin is shown in Table 1.
b) Initial pH was 5.0.
c) Agar was add to the isolation medium for sprinkled soil plate method.
d) $(NH_4)_2SO_4$ 0.20% was used instead of $(NH_2)_2CO$ 0.38% for the production medium in jar fermenter.

Determination of cell production in shaking culture After the culture broth had been centrifuged, both sedimented cells and floating cells with unassimilated *n*-paraffin were collected using a pre-weighed filter and washed first with acetone and then *n*-hexane. The filter containing cells was dried at 120°C for 2 hr and weighed.

Determination of cell yield in jar fermenters Cultivation was stopped after the *n*-paraffin had completely been used. All of the cells in the fermenter were centrifuged, washed with water, dried and weighed. Cell yield was calculated as follow:

$$\text{Cell yield} = \frac{\text{Weight of dried cells (g)}}{\text{Weight of } n\text{-paraffin added (g)}} \times 100 \ (\%)$$

Determination of protein content of cells Protein content was calculated by multiplying the nitrogen content by 6.25.

Identification of yeasts The experimental methods were mainly those described by Lodder and Kreger-van Rij,[5] Lodder *et al.*[6] and Iizuka and Goto.[7] The results were discussed according to the system of Lodder *et al.*[6]

Results and Discussion

Screening of n-paraffin assimilating yeasts From soil samples, 284 *n*-paraffin assimilating yeast strains grown at 37°C were isolated. Among them, 21 strains showed almost the same or better growth in *n*-paraffin medium at 37°C by shaking culture than *C. rugosa* JF-114 at 30°C. The cell production in shaking culture, cell yields against *n*-paraffin added to the medium in jar fermenter studies, and protein content of the cells are shown in Table 3.

Strain MT-Y-8 showed the best cell yield and highest protein content among the 21 strains. As the strain is considered to be useful for industrial cell production from *n*-paraffin, a forthcoming paper will describe the growth conditions and the posibility of industrial yeast cell production of this strain.[8,9]

Although the cell yield and protein content of strain MT-Y-1 were both lower than those of the strain MT-Y-8, this strain is worthy of attention because it produced well developed mycelial cells when grown in *n*-paraffin medium. This means easy separation of the cells from cultured broth. Strains MT-Y-2 and MT-Y-3 showed almost the same cell yields as strain MT-Y-8. However, the protein content of the cells was very low. There were 3 strains which were very foamy during fermentation and 2 strains which produced extraodinarily bad odor. Other strains had no special characteristics from the industrial view point.

Identification of the 21 *n*-paraffin assimilating yeast strains which grew well at 37°C These 21 strains reproduced by multilateral budding and did not form balistospores, ascospores, teliospores or arthrospores. The vegetative cells were not triangular or "ogival". They did not produced strong acetic acid from glucose or starch-like compounds. The streak cultures were not pigmented. Pseudomycelia were formed. Some of the strains formed true mycelia. They were all confirmed to belong to the genus *Candida*. According to the morphological and physiological studies, these 21 strains were classified into 8 species including one new species. However, more than half of them (12 strains) were identified as *C. tropicalis*. The other 9 strains were identified as *C. solani* (2 strains), *C. albicans* (2 strains), *C. krusei* (1 strain), *C. intermedia* (1 strain), *C. rugosa* (1 strain), *C. parapsilosis* (1 strain), and a new species of genus *Candida*. The species name of each strain is also shown in Table 3.

The properties of the 12 strains which were identified as *C. tropicalis* agreed well with

Table 3. Results of screening

Strain	Species	Cell production (g/100 ml)	Cell yield (%)	Protein content (%)	Notes
MT-Y-1	*Candida tropicalis*	1.33	88.0	56.3	Mycelial cell
MT-Y-2	*C. solani*	1.30	94.6	39.8	
MT-Y-3	*C. solani*	1.21	93.3	44.7	
MT-Y-4	*C. tropicalis*	1.39	76.3	56.0	
MT-Y-5	*C. tropicalis*	1.31	73.5	56.8	
MT-Y-6	*C. tropicalis*	1.12	86.0	56.9	
MT-Y-7	*C. tropicalis*	1.35	76.4	53.6	
MT-Y-8	*C. kofuensis*	1.38	95.5	61.8	
MT-Y-9	*C. intermedia*	1.32	83.0	53.1	
MT-Y-10	*C. tropicalis*	1.32	70.3	54.1	
MT-Y-11	*C. tropicalis*	1.25	82.0	57.4	Foamy
MT-Y-12	*C. krusei*	1.16	84.3	58.8	
MT-Y-13	*C. tropicalis*	1.12	71.3	51.3	Bad odor
MT-Y-14	*C. rugosa*	1.60	76.2	50.6	Foamy
MT-Y-15	*C. tropicalis*	1.16	73.0	49.9	
MT-Y-16	*C. parapsilosis*	1.32	73.0	55.4	
MT-Y-17	*C. tropicalis*	1.41	73.4	50.7	Foamy
MT-Y-18	*C. albicans*	1.35	79.6	51.6	
MT-Y-19	*C. tropicalis*	1.24	79.0	50.9	
MT-Y-20	*C. tropicalis*	1.26	75.7	51.1	Bad odor
MT-Y-21	*C. albicans*	1.26	81.0	53.4	
JF-114	*C. rugosa*	1.18	87.8	54.6	

All the strains were cultivated at 37°C except strain JF-114. JF-114 was cultivated at 30°C.

the standard description given by Lodder et al.[6] except for strain MT-Y-1. The physiological properties of strain MT-Y-1 agreed approximately with the standard description, but some of the morphological properties differed from it and from those of the other *C. tropicalis* strains. Namely, this strain had a distinctively tomentose appearance over the whole colony. The strain MT-Y-1 was also characterized by well developed mycelia when grown in *n*-paraffin medium in contrast to the other *C. tropicalis* strains.

Strain MT-Y-8 could not be identified as any known species described in the literature. Although the physiological and morphological properties of this strain seemed to be similar to those of *Candida tenuis*, some significant differences were found in the fermentation and the assimilation of carbon sources and the maximum temperature for growth. It was, therefore, considered to be a new species of *Candida* and we propose to name it *Candida kofuensis* as it was isolated in Kofu City.

Description of *Candida kofuensis*

Candida kofuensis Goto *et* Asai, nov. sp.

Cultura in extracto malti; Ad 25°C, post 3 dies cellulae globosae, longi-ovales ver elongatae, 1.5–2.5 × 1.5–3.5 μm, singulae, binae et catenatae, mycelium verum formatur; pellicula et sedimentum formatur.

Cultura in agaro malti: Cultura in striis post unum mensem ad 17°C mucose et

plicata, color cremeus, pagina rugosa et opaca, margo integer, pseudomycelium et mycelium verum formatur.

Fermentatio: Glucosum exigue.

Assimilatio originum carbinu: Glucosum, galactosum, L-sorbosum, maltosum, sucrosum, cellobiosum, trehalosum, lactosum, melezitosum, inulinum, D-xylosum, L-rhamnosum, glycerolum, ethanoleum, i-erythritolum, adonitolum, dulcitolum, D-mannitolum, D-sorbitolum, alphamethylglucosidum, salicinum, glucono-δ-lactonum, calcium-2-ketogluconatum, kalium-5-ketogluconatum, acidum lacticum, acidum succinicum, acidum citricum et i-inositolum assimilantur, at non melibiosum, raffinosum, amylum solubile, L-arabinosum, D-arabinosum, nec D-ribosum.

Kalium nitricum assimilatur, amylosum non formatur, arbutinum non finditur.

Temperatura maxima crescentiae 46°C.

Habitat in solo.

Typus: in solo, Japonia, Yamanashi Prov., Kofu, 9.i.1969, cultura in tobo no. MT–Y–8.

Growth in malt extract: After 3 days at 25°C, cells are globose long-oval to elongate, 1.5–2.5 × 1.5–3.5 μm, single, in short chains. True mycelium is also formed. Pellicle and sediment are formed.

Growth on malt agar: After one month at 17°C, the streak-culture is cream-colored, dull mucoid, folded and entire at the margin.

Culture on glass slide: The pseudomycelium is well developed, true mycelium is also formed.

Fermentation: D-Glucose is fermented. D-Galactose, saccharose, maltose, lactose and raffinose are not fermented.

Assimilation of carbon compounds: D-Glucose, galactose, L-sorbose, maltose, sucrose, cellobiose, trehalose, lactose, melezitose, inulin, D-xylose, L-rhamnose, glycerol, ethanol, *i*-erythritol, adonitol, dulcitol, D-mannitol, D-sorbitol, α-methyl-D-glucoside, salicin, glucono-δ-lactone, Ca-2-keto-gluconate, K-5-keto-gluconate, DL-lactic acid, succinic acid, citric acid and *i*-inositol are assimilated. Melibiose, raffinose, soluble starch, L-arabinose, D-arabinose and D-ribose are not assimilated.

Assimilation of nitrogen compounds: Peptone, ammonium sulfate, asparagine and urea are assimilated. Potassium nitrate is not assimilated.

Splitting of arbutin: Negative.

Reaction in litmus-milk: No change.

Production of starch-like compounds: Negative.

Gelatin liquefaction: Negative.

Vitamin requirement for growth: None.

Temperature range for growth: 17–46°C.

Collection examined: Soil sample from Kofu City, Yamanashi Prefecture.

Culture MT–Y–8 is the type strain of this species.

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